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Cytoprotection of human endothelial cells from menadione cytotoxicity by caffeic acid phenethyl ester: The role of heme oxygenase-1

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ABSTRACT

Caffeic acid phenethyl ester (CAPE), derived from various plant sources, has been shown to ameliorate ischemia/reperfusion injury *in vivo*, and this has been attributed to its ability to reduce oxidative stress. Here we investigated the cytoprotection of CAPE against menadione-induced oxidative stress in human umbilical vein endothelial cells (HUVEC) to evaluate potential gene expression involvement. CAPE exhibited dose-dependent cytoprotection of HUVEC. A gene screen with microarrays was performed to identify the potential cytoprotective gene(s) induced by CAPE. Heme oxygenase-1 (HO-1) was highly upregulated by CAPE and this was confirmed with reverse transcriptase polymerase chain reaction (RT-PCR) and western blotting. Inhibition of HO-1 activity using the HO-1 inhibitor tin protoporphyrin IX (SnPPIX), resulted in loss of cytoprotection. Carbon monoxide, one of HO-1 catabolic products appeared to play a small role in CAPE protection. Caffeic acid, a potential metabolite of CAPE with similar free radical scavenging ability, however, didn't show any cytoprotective effect nor induce HO-1. These findings suggest an important role of HO-1 induction in CAPE cytoprotection against oxidant stress, which may not relate to CAPE structural antioxidant activity nor to its traditional enzymatic activity in decomposing heme but to a yet to be determined activity.
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1. Introduction

Interruption in the flow of blood to a tissue leads to tissue ischemia, which is a major cause of morbidity and death contributing to disease processes such as heart disease, stroke, cancer, and traumatic injury. Failure to quickly reestablish flow to ischemic tissue results in tissue death, but even timely return to normal flow is detrimental because reintroduction of oxygen to ischemic tissue results in ischemia/reperfusion injury to the tissue. Caffeic acid phenethyl ester (CAPE), a plant-derived polyphenolic compound, has been shown to protect different organs from ischemia/reperfusion induced injury in various animal models (Ilhan et al., 1999; Esrefoglu et al., 2005; Ozer et al., 2005; Parlakpinar et al., 2005; Ozyurt et al., 2006).

CAPE, present in honeybee propolis, may be responsible for many biological activities including antitumor (Huang et al., 1996), antiviral (Fesen et al., 1994), immunomodulatory (Park et al., 2004), anti-inflammatory (Fitzpatrick et al., 2001), and antioxidant effects (Hsu et al., 2005). Recently, CAPE has been demonstrated to ameliorate ischemia/reperfusion injury *in vivo* in spinal cord (Ilhan et al., 1999), skeletal muscle (Ozyurt et al., 2006), and myocardium (Parlakpinar et al., 2005), and to protect remote organs such as testes and kidney from myocardial ischemia/reperfusion (Esrefoglu et al., 2005; Ozer et al., 2005). While its protective activity has been attributed to its known antioxidant ability, CAPE has also been shown to affect transcriptional activity, which may be similar to the cytoprotective effect of other polyphenolic antioxidants like curcumin and resveratrol (Chen et al., 2005; Motterlini et al., 2000; Scapagnini et al., 2002). The result presented here indicates that cytoprotection in human endothelial cells by CAPE involves transcriptional activation of heme oxygenase-1 (HO-1).

HO-1 is best known as the inducible isoform of heme oxygenase and the rate-limiting enzyme in the catabolism of heme into carbon monoxide (CO), biliverdin (which is rapidly converted to bilirubin by biliverdin reductase), and free iron (which is sequestered to ferritin, an iron-binding protein). HO-1 has been demonstrated to be a protective gene in various cell types due to its anti-inflammatory, anti-apoptotic and anti-proliferative properties (Lee et al., 1996; Willis et al., 1996; Brouard et al., 2000), which may support its role in attenuating ischemia/reperfusion injury. It has been reported that the induction of HO-1 or its heme-degrading products protect different organs from ischemia/reperfusion injury *in vivo* including gut, intestine, liver, kidney and heart (Akamatsu et al., 2004; Attuwaybi et al., 2004; Berberat et al., 2003; Clark et al., 2000; Fondevila et al., 2003; Hammerman et al.,

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2002; Nakao et al., 2005; Salom et al., 2007). In addition, the "knocking down" of HO-1 exacerbated ischemia/reperfusion-induced lung and myocardial injury (Liu et al., 2005; Zhang et al., 2004). Recently, human HO-1 gene delivery using an adenovirus vector has been shown to be a feasible strategy for protecting heart, liver, and skeletal muscle in a rat ischemia/reperfusion model (Pachori et al., 2004). However, the requirement for production of heme-degrading products as the cytoprotective agents has recently been called into question (Hori et al., 2002; Sheftel et al., 2007).

Endothelial cells, which line all blood vessels, appear to play an important role during ischemia/reperfusion injury. They are known to respond to various stimuli, in part by changing the gene expression for cytokines, adhesion molecules, procoagulation factors, and other proteins (Granger et al., 2004; Minami et al., 2004). Our previous studies showed that CAPE protects human umbilical vein endothelial cells (HUVEC) against oxidative stress induced by menadione (Wang et al., 2006). As one of the simplest quinones, menadione has been widely studied as a model for evaluating the cellular effects of oxidative stress in endothelial cells, including induction of apoptosis (Kossenjans et al., 1996; Warren et al., 2000). The major mechanism of endothelial cell toxicity caused by menadione is the intracellular production of reactive oxygen species by redox cycling, where one electron reduction of O_2 by the semiquinone form of menadione generates superoxide. Superoxide dismutates to form hydrogen peroxide, which then participates in the Fenton reactions producing hydroxyl radicals. Because the generation of reactive oxygen species is a major outcome of ischemia/reperfusion injury, menadione-induced endothelial cell injury was used to simulate ischemia/reperfusion injury *in vitro*. The purpose of our present study was to investigate the mechanism of CAPE protection against reactive oxygen species-mediated injury *in vitro*. We hypothesized that heme oxygenase-1 induction is the key component in CAPE protection against oxidative stress.

2. Materials and methods

2.1. Materials

CAPE was purchased from Cayman Chemical (Ann Arbor, MI, USA). Caffeic acid, bilirubin, menadione sodium bisulfite (menadione), and DMSO were purchased from Sigma (Saint Louis, MO, USA). Biliverdin hydrochloride and Sn(IV) protoporphyrin IX dichloride (SnPPIX) were purchased from Frontier Scientific (Logan, UT, USA). 5-(and-6)-chlormethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) was obtained from Invitrogen (Carlsbad, CA, USA).

2.2. Cell culture

First passage HUVEC (Cascade Biologics, Portland, OR, USA) were cultivated on 1% gelatin-coated 75-cm^2 culture flasks (Corning Incorporated, Corning, NY, USA) in Medium 200 supplemented with 2% fetal calf serum, penicillin (100 units/ml), streptomycin (100 units/ml), and Fungizone (0.25 μg/ml) supplied by Cascade Biologics (Zhao et al., 2001). The cells were cultivated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 with medium changes every 2 days until confluent. Prior to an experiment, HUVEC were subcultivated with Trypsin/EDTA onto 1% gelatin-coated Costar® multiplates (Corning Incorporated, Corning, NY, USA) at 5000 cells/cm^2, grown to confluence, and kept for 72 h to produce a quiescent cell layer. On the day before the experiment, the medium was changed. Only the second through fifth population doublings of cells were used.

2.3. Cell viability assay

Cell viability was assessed at 24 h after initiation of treatment using Alamar Blue™ (Biosource International, Camarillo, CA, USA), which is converted to a fluorescent compound in amounts proportional to the number of viable cells (Larson et al., 1997). The cells were incubated for 2 h at 37 °C with culture medium containing 10% Alamar Blue™. After incubation, fluorescence was measured at 545 nm excitation and 590 nm emissions using SpectraMAX M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA). HUVEC were regularly observed under phase contrast microscopy.

2.4. Menadione cytotoxicity assay

Menadione bisulfite (0.5 M) was dissolved in phosphate buffered saline (PBS) (Gao et al., 2001) and diluted with medium before being added to the plate wells. Due to differences in cellular responses to menadione, each group of pooled HUVEC was initially assessed for a dose of menadione which is close to its toxicity producing 80–90% cell death. Cells were incubated in 5 to 38 μM menadione depending on the sensitivity of a given set of cells for 24 h followed by Alamar Blue viability assay.

2.5. In vitro cytoprotection assay

CAPE, biliverdin, bilirubin, and caffeic acid were dissolved in DMSO and diluted 1000-fold with medium before addition of serial dilutions to the 48-well culture plates. To assess protection against oxidative stress, confluent HUVEC were initially pretreated with CAPE, biliverdin, bilirubin, and caffeic acid at various concentrations for 6 h, and the cytotoxic dose of menadione chosen from menadione cytotoxicity assay was then given to the HUVEC in the presence of testing compounds. After 24 h incubation, cell viability was measured using the Alamar Blue assay.

2.6. Total RNA isolation

Total RNA was extracted from cultured HUVEC grown in 6-well multiplates with TRI™ reagent according to the manufacture's instructions (Molecular Research Center, Cincinnati, OH, USA). RNA yield was quantified using NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and its quality was assessed by electrophoresis on 1% agarose gels containing 1:1000 SYBR Gold in the loading buffer (Invitrogen, Carlsbad, CA, USA).

2.7. Polyacrylamide gel electrophoresis, and western blotting

Protein was extracted from the HUVEC grown on gelatin coated 24-well multiplates after incubation with CAPE (17.6 μM) for 24 h, by addition of 50 μl of lysis buffer (NOVEX, San Diego, CA, USA) containing 10 mM tris (carboxyethyl) phosphine hydrochloride (Sigma, St Louis, MO, USA). Fifteen microliters, containing approximately 5 μg of protein, from each treatment were run on NuPage 4–12% bis-tris gels (Invitrogen) and then transferred to a nitrocellulose membrane (Invitrogen). After blocking in 0.2% I-Block (Tropix, Bedford, MA, USA), 0.1% Tween-20 (Sigma) and 0.1% thimerosal (Sigma) in PBS, the blots were then incubated with a rabbit anti-rat HO-1 antibody (Assay Designs Inc., Ann Arbor, MI, USA; 1:5000) for 2 h. Rabbit ABC alkaline phosphatase reagents (Vector Laboratories, Burlingame, CA, USA) were used to label the bands and the alkaline phosphotase visualization was accomplished with nitro blue tetrazolium chloride (NBT) and 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) (Invitrogen) until the bands developed. Quantitative analysis was performed with NIH Images (NIH, USA) on blots scanned into the computer. Following staining for HO-1, blots were stripped and reprobed with mouse anti-actin (Abcam, Cambridge, MA, USA) as a control for equal loading of protein.

2.8. Gene expression analysis

Microarrays containing ~22,000 human genes per array were produced by spotting oligonucleotides (Qiagen/Operon Human

ArrayReady Oligo Set V 2.0, Alameda, CA, USA) onto epoxy-coated slides (Cel Associates, Pearland, TX, USA). A GeneMachines Omni Accent Gridder (GeneMachines, San Carlos, CA, USA) and SMP-3 pins from Telechem International (Sunnyvale, CA, USA) were applied to print the microarrays. Microarrays were blocked with 1% BSA in 3× SSC and 0.1% SDS for 1 h at 50 °C before use.

For gene expression analysis, RNA extracted from CAPE-treated (17.6 μM) and DMSO-treated HUVEC (0.1%) following a 6-h incubation were compared to human universal reference RNA (Stratagene, La Jolla, CA, USA). cDNA targets were generated with the 3DNA Array 900 kit (Genisphere Inc., Hatfield, PA, USA) according to the manufacture's instructions. The high-resolution scans (10 μm) were acquired with a GenePix scanner 4000B (Axon Instruments, Inc., Union City, CA, USA) and tabulated with GenePix pro 5.1 software. To determine intra-assay variability, each condition (3 replicates) was individually labeled and compared to a universal reference of pooled RNA that was labeled as a pool sufficient for 6 microarrays. Statistical analysis for differences between treatment groups was performed using BRB Array Tools (Biometric Research Branch, National Cancer Institute, USA, http://linus.nci.nih.gov/BRB-ArrayTools.html).

2.9. Quantitative real-time RT-PCR

One μg of total RNA from the same samples used for microarray analysis was converted to cDNA using random primers and Superscript III reverse transcriptase according to the manufacture's instruction (Invitrogen™ life Technology, Carlsbad, CA, USA). Real-time PCR was performed on a LightCycler™ thermal cycler (Idaho Technology, Salt Lake City, UT, USA) with Roche LightCycler® TaqMan Master for heme oxygenase-1 and β-actin (Roche Diagnostics, Indianapolis, IN, USA). HO-1 and β-actin primer sets were from TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). HO-1 gene was normalized to the expression level of β-actin gene for each sample. Relative quantification was acquired by comparative C_T method.

Table 1
CAPE-regulated significant alteration of gene expression in HUVEC

Gene category	Gene description	Fold change
Detoxification	**Heme oxygenase (decycling) 1**	**8.245**
Molecular chaperone	Heat shock protein 90 kDa beta (Grp94), member 1	2.832
	Heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa)	3.954
Growth factor	KIT ligand	4.727
	Prostate differentiation factor	4.625
	Endothelial cell-specific molecule 1	2.878
Transporter	Solute carrier family 3, member 2	3.187
	Solute carrier family 30 (zinc transporter), member 1	2.492
	Solute carrier family 39 (zinc transporter), member 14	2.834
	START domain containing 4, sterol regulated	2.705
Binding	Selenoprotein K (selenium binding)	3.399
	Nucleobindin 2 (calcium ion binding)	3.146
	Asp (abnormal spindle)-like (calmodulin binding)	0.285
	Phorbol-12-myristate-13-acetate-induced protein 1 (protein binding)	2.508
Transcription regulator	BTG family, member 2	2.572
	DNA-damage-inducible transcript 3 (P38 MAPK signaling pathway)	4.717
Apoptosis	BCL2/adenovirus E1B 19 kDa interacting protein 3	3.490
Cytokine	Interleukin 8	3.479
Kinase	Eukaryotic translation initiation factor 2-alpha kinase 3	2.914
Transferase	Phosphatidylinositol glycan, class A	2.942
Others	Spindle pole body component 25 homolog (*S. cerevisiae*)	0.313
	Transmembrane protein 50B	3.451
	CDNA FLJ14162 fis, clone NT2RM4002504	2.696

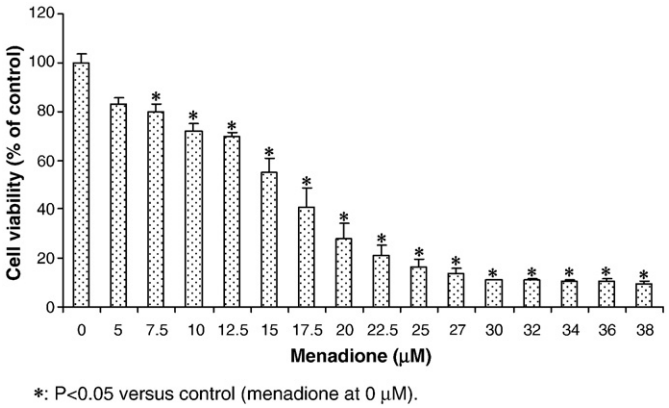

Fig. 1. Toxicity of menadione in HUVEC. Menadione caused a dose-dependent reduction of viability in HUVEC. Values are presented as means with standard deviations (n=3). The dose of menadione at 25 μM was used in the CAPE cytoprotection assay.

2.10. Heme oxygenase-1 inhibition with SnPPIX

HUVEC were pretreated with 17.6 μM CAPE and different doses of HO-1 inhibitor SnPPIX (Abuarqoub et al., 2006), respectively, for 6 h before exposing to menadione for 24 h. SnPPIX was dissolved in 0.1 M NaOH and dilute 1000-fold with medium before being added to the 48-well culture plates. Cell viability was measured using the Alamar Blue assay.

2.11. Carbon monoxide exposure

HUVEC were exposed to 1% CO and 5% CO_2 in air (Matheson-TriGas, Kyle, TX, USA) in sealed incubator chambers (Billups-Rothenberg Inc, Del Mar, CA, USA). Cultures identically treated but exposed to 95% air 5% CO_2 served as control. After a 10-min purging of either CO or air, HUVEC were incubated for 2 h. Various concentrations of menadione were then added to the culture media and the culture plates were returned to the chambers. After 10 min of purging with 1% CO or air, HUVEC were incubated for additional 24 h in sealed chambers. The cell culture chambers were humidified and maintained at 37 °C. Cell viability was measured using the Alamar Blue assay.

2.12. Assay for measuring reactive oxygen species

Intracellular production of reactive oxygen species was evaluated by exposing HUVEC to 20 μM CAPE, caffeic acid, or 0.1% DMSO and

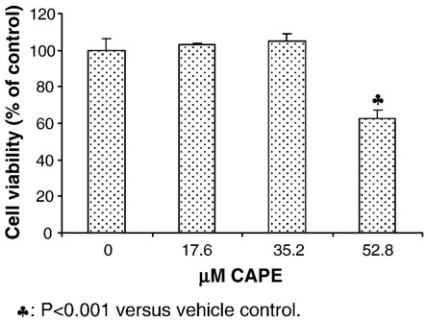

Fig. 2. Cytotoxicity of CAPE to HUVEC. Values are presented as means with standard deviations (n=3). Cell viability is shown as percent of control, and less than 90% was considered toxic. Therefore, CAPE at 52.8 μM was considered toxic.

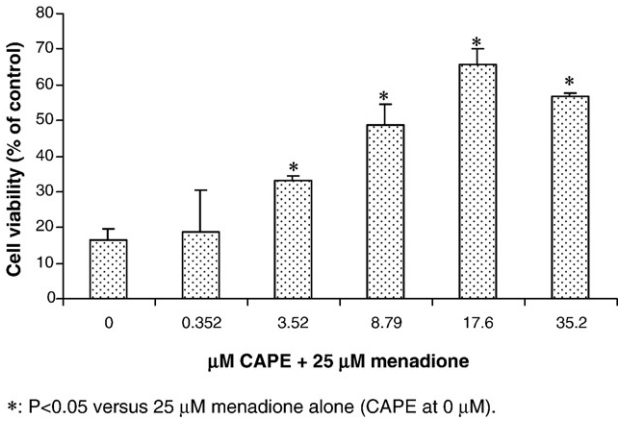

Fig. 3. Cytoprotection against menadione-induced injury in HUVEC by CAPE. Menadione was used at 25 μM. Values are presented as means with standard deviations (n=3). CAPE showed dose-dependent cytoprotection against menadione. CAPE at 17.6 μM recovered HUVEC significantly to about 65% from menadione-induced injury (P=0.001).

fluorescent probe 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) (Jones et al., 2002). HUVEC were seeded onto 96-well plates and grown to confluence. HUVEC were rinsed twice with prewarmed Hanks' HEPES buffer prepared with Hanks' balanced salt solution (without sodium bicarbonate or phenol red; Invitrogen) and 10 mM HEPES buffer (Sigma), and incubated for 30 min at 37 °C in the same solution with CM-H_2DCFDA (8 μM) premixed in Hanks' HEPES buffer. HUVEC were then washed twice with prewarmed Hanks' HEPES buffer and loaded with the testing compounds dissolved in Hanks' HEPES buffer. The fluorescence released from the cells was recorded immediately at 480 nm (excitation) and 520 nm (emission) using SpectraMAX M2 microplate reader at different time intervals over a 2-h period while the HUVEC were incubated at 37 °C within the plate reader. The fluorescent signals were normalized to the initial readings at time zero. The relative fluorescence was proportional to the production of reactive oxygen species.

2.13. Statistical analysis

Data are presented as the mean±standard deviation. Differences between or among the groups were analyzed using the independent samples T test or one-way analysis of variance combined with Tukey (equal variances assumed) or Games-Howell (equal variances not assumed) test through SPSS statistical software. A difference of p value<0.05 was considered significant. Each experiment was repeated at least 3 times and a representative experiment is presented.

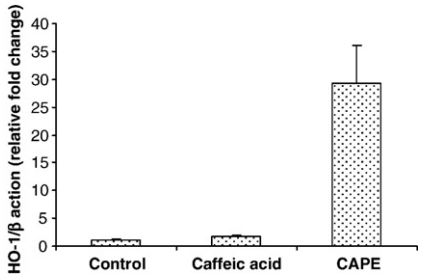

Fig. 4. Heme oxygenase-1 gene induction by CAPE and caffeic acid in HUVEC by relative quantitative real-time RT-PCR. Heme oxygenase-1 expression was highly increased (29-fold) in CAPE group compared to DMSO control. Caffeic acid group didn't induce HO-1 significantly compared to the control group. Values are presented as means with standard deviations (n=3).

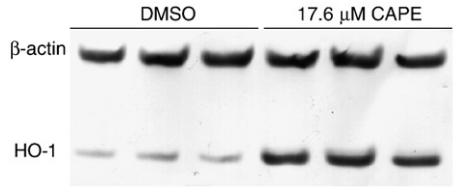

Fig. 5. Effect of CAPE on HO-1 protein expression in HUVEC by western blot. The induction of HO-1 protein was determined in HUVEC after incubating with 17.6 μM CAPE and DMSO control for 24 h.

For microarray analysis with BRB Array Tools, genes were excluded if flagged. After lowess smooth normalization, a two-class comparison was performed to identify genes that were differentially expressed using a random-variance t-test. Genes were considered to be significantly altered in their expression if the P value<0.001. The common genes significantly up-regulated between two independent experiments were identified by intersecting the two individual genelists in BRB Array Tools. The mean fold changes from two independent experiments are presented in Table 1. The series of datasets discussed herein can be accessed at the Gene Expression Omnibus (GEO) website under the submission number GSE10465 which includes two subset series (GSE10413 and GSE10429) corresponding to two independent experiments (http://www.ncbi.nlm.nih.gov/geo/).

3. Results

3.1. Protective effects of CAPE against menadione-induced oxidative stress in human endothelial cells

The oxidant stress model of HUVEC produced by menadione-generated reactive oxygen species is shown in Fig. 1. Menadione induced a dose-dependent decrease in the viability of HUVEC as demonstrated by the Alamar Blue assay.

CAPE cytotoxicity was examined at 17.6, 35.2, and 52.8 μM and is shown in Fig. 2. Because 52.8 μM of CAPE caused more than 10% cell death, the maximum dose of CAPE used for cytoprotection testing was 35.2 μM. CAPE protected HUVEC against menadione-induced ischemia/reperfusion-like injury in a dose-dependent fashion (Fig. 3). At 17.6 μM, CAPE had the maximum cytoprotective effect against menadione-induced toxicity, showing 65% HUVEC recovery compared to about 15% cell viability after menadione exposure alone.

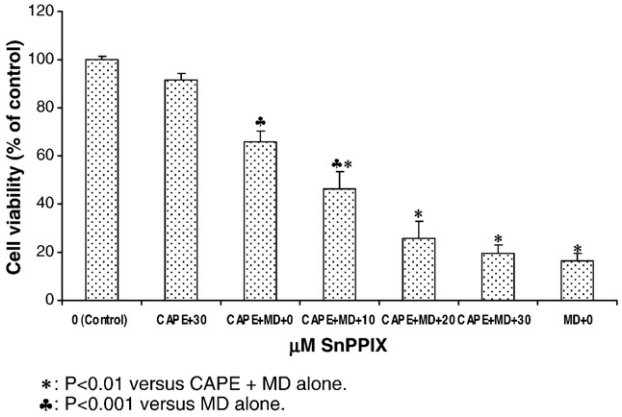

Fig. 6. The effect of HO-1 inhibitor SnPPIX on 17.6 μM CAPE cytoprotection against menadione (MD)-mediated oxidative injury in HUVEC. Values are presented as means with standard deviations (n=3). SnPPIX exerted dose-dependent suppression on 17.6 μM CAPE protection against 25 μM MD-induced oxidative injury. CAPE plus 30 μM SnPPIX alone was not toxic.

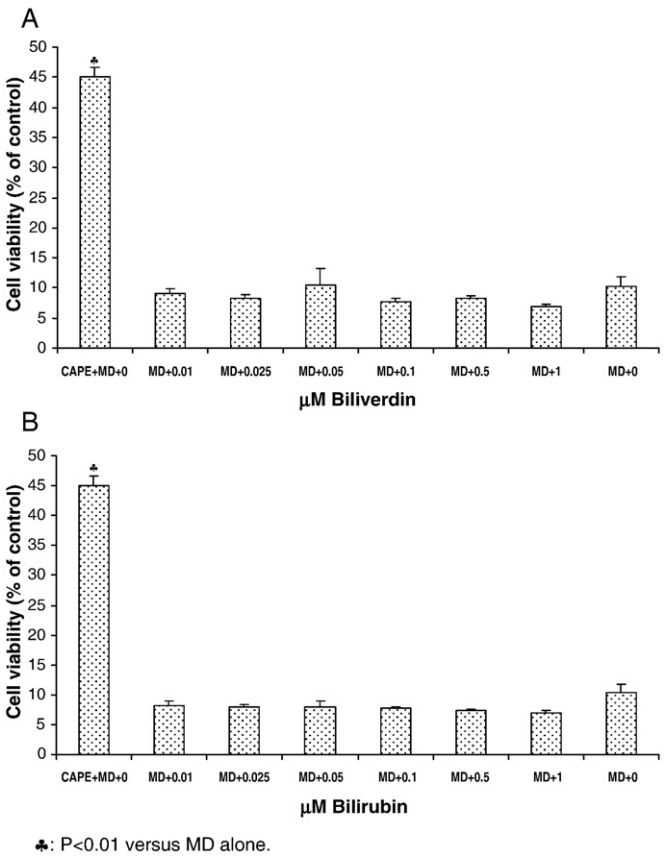

Fig. 7. Cytoprotective effects of biliverdin (A) and bilirubin (B) testing compared to 17.6 μM CAPE in HUVEC against oxidative stress induced by 30μM menadione (MD). Values are presented as means with standard deviations (n=3). Neither biliverdin nor bilirubin from 0.01 μM to 1 μM demonstrated protection.

3.2. Heme oxygenase-1 induction by CAPE in HUVEC

To investigate the mechanism behind CAPE cytoprotection at the transcriptional level, gene expression analysis was performed using oligonucleotide microarrays. Of the 22,000 human genes examined from two independent experiments, 23 genes were statistically significantly up-regulated during the early response (6 h) to CAPE (Table 1). Among them, HO-1 was highly induced (8.25-fold) by CAPE compared to DMSO control. To validate this particular microarray screening result, quantitative real-time RT-PCR was performed at 6 h and western blotting was performed for HO-1 at 24 h. The results confirmed that the HO-1 gene was upregulated about 29-fold (Fig. 4) while its protein product was elevated about 6-fold (Fig. 5).

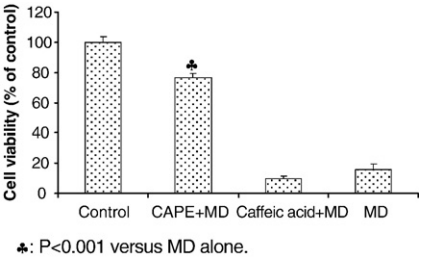

Fig. 9. Cytoprotective activities of caffeic acid versus CAPE at 20 μM in 22.5 μM menadione (MD)-treated HUVEC. Values are represented as means with standard deviations (n=3). Caffeic acid was not cytoprotective in HUVEC against MD-induced cytotoxicity.

3.3. Heme oxygenase-1 inhibitor SnPPIX effects on CAPE cytoprotection

To test whether the cytoprotective effects of CAPE were dependent on HO-1 activity, HO-1 activity was blocked by its competitive inhibitor SnPPIX. Coincubating with varying concentrations of SnPPIX and 17.6 μM CAPE resulted in reduction of cytoprotection against menadione dose-dependently (Fig. 6).

3.4. The effects of biliverdin and bilirubin

To further elucidate the role of HO-1 in CAPE cytoprotection, we tested heme catabolism products biliverdin and bilirubin. They have been reported to be potent antioxidants against peroxyl radicals (Stocker et al., 1987), which may contribute to the protective effect of HO-1. In this study, biliverdin and bilirubin ranging from 0.01 μM to 1 μM showed no protective effect (Fig. 7), and these doses did not appear to be toxic alone in HUVEC (data not shown).

3.5. The effects of carbon monoxide

To determine if CO could account for the cytoprotective effect of HO-1, HUVEC were exposed to various concentrations of menadione in the presence or absence of 1% CO and cell viability assessed by the Alamar Blue assay. Cells in the presence of CO showed better survivability at low doses of menadione (5–10 μM) (Fig. 8).

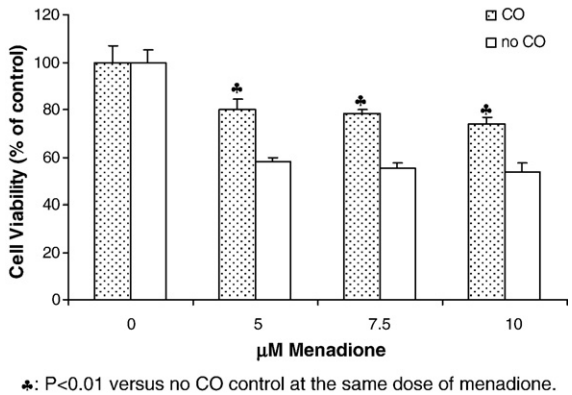

Fig. 8. The cytoprotective effect of 1% CO against low doses of menadione-induced oxidative damage in HUVEC. Values are represented as means with standard deviation (n=3). About 20% more cell viability was observed in 1% CO-treated HUVEC (black-spot filled column) than control (white column).

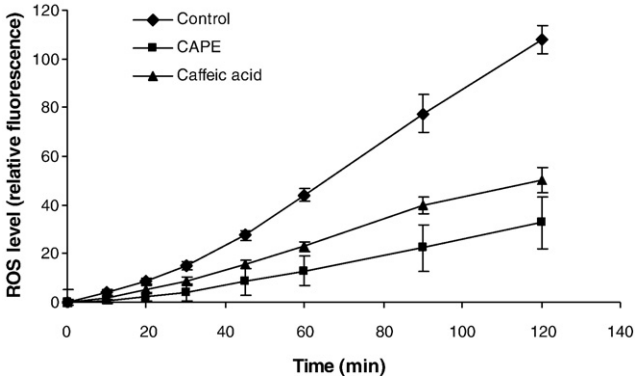

Fig. 10. Direct antioxidant effect of CAPE and caffeic acid at 20 μM on intracellular production of reactive oxygen species (ROS). Values are represented as means with standard deviations (n=4). Both CAPE and caffeic acid served as antioxidants by reducing the basal level of reactive oxygen species in 2 h due to their structural-related free radical scavenging abilities.

3.6. The effect of caffeic acid with similar structural antioxidant activity to CAPE

The structural feature responsible for the free radical scavenging activities of CAPE is the two adjacent hydroxyl groups on its catechol moiety (Chen and Ho, 1997). We asked if the beneficial effect of CAPE was shared by caffeic acid, a potential metabolite of CAPE with the same antioxidant functional group. The data in Fig. 9 showed no cytoprotective effect of caffeic acid compared to CAPE at the same molar amount (20 μM). RT-PCR data indicated that HO-1 was not induced by caffeic acid either (Fig. 4). The determination of intracellular generation of reactive oxygen species indicated that both CAPE and caffeic acid behaved as similarly as antioxidants in HUVEC scavenging free radicals (Fig. 10).

4. Discussion

Ischemia/reperfusion injury occurs in cardiovascular disease, surgical procedures, organ transplantations and following traumatic injury. The human endothelial cell plays an important role in this type of injury and it is important to protect it from further dysfunction and restore its normal function as soon as possible after injury. Polyphenolic compounds such as CAPE may provide some amelioration of ischemia/reperfusion injury at the level of the endothelial cells. To better understand the potential protective mechanism of CAPE, we developed an *in vitro* model of menadione-induced oxidative stress in HUVEC. This study demonstrated that CAPE provided dose-dependent cytoprotection of HUVEC against menadione-caused oxidative injury. The antioxidant activity of CAPE has been proposed to account for its beneficial effect in ischemia/reperfusion injury (Esrefoglu et al., 2005; Ilhan et al., 1999; Ozer et al., 2005; Ozyurt et al., 2006; Parlakpinar et al., 2005). As a polyphenolic compound, CAPE maintains the catechol ring with 3,4-dihydroxyl configuration, which can directly scavenge free radicals. In addition, CAPE may also exert its protective effect via transcriptional activation as do some other polyphenols (Chen et al., 2005; Motterlini et al., 2000; Scapagnini et al., 2002). To investigate its mechanism of cytoprotection at the transcriptional level, gene expression analysis was performed. It was determined that HO-1 was highly induced by CAPE in HUVEC with confirmation by RT-PCR and western blot.

HO-1 induction has been shown to be beneficial in ischemia/reperfusion injury *in vivo* (Pachori et al., 2004; Salom et al., 2007). Several mechanisms of HO-1 protection against ischemia/reperfusion injury have been proposed: 1) metabolism of heme, the toxic cellular component from disposed heme proteins; 2) consumption of excessive O_2 during ischemia/reperfusion injury in the process of heme degradation by HO-1; and 3) cytoprotection through heme catabolizing products such as biliverdin/bilirubin, ferrous iron/ferritin, and carbon monoxide (Katori et al., 2002). To further elucidate the role of HO-1 induction in CAPE cytoprotection, HO-1 activity was suppressed by using HO-1 inhibitor SnPPIX. The cytoprotective effect of CAPE against menadione-induced cytotoxicity was abrogated in a concentration-dependent fashion with the co-incubation of non-toxic doses of SnPPIX. This result indicated that HO-1 induction plays an important role in CAPE cytoprotection. Since heme degradation by HO-1 results in biliverdin, bilirubin, and CO production and all have been reported to ameliorate ischemia/reperfusion induced injury in different organs *in vivo* (Akamatsu et al., 2004; Clark et al., 2000; Fondevila et al., 2003; Hammerman et al., 2002), we examined their cytoprotective activities. BR as low as 10 nM can protect cells against oxidative stress induced by 100 μM hydrogen peroxide, which seems to function through the conversion from biliverdin to bilirubin by the action of biliverdin reductase (Baranano et al., 2002). Therefore, we tested the cytoprotective effects of biliverdin to bilirubin ranging from 0.01 to 1 μM, but no protection was observed compared to CAPE. Generation of CO by HO-1 might explain CAPE cytoprotection evidenced from the suppression of endothelial cell apoptosis by 1% exogenous CO exposure (Brouard et al., 2000). Mishra recently reported that carbon monoxide provided cytoprotection in ischemic lungs by interrupting mitogen activated protein kinase expression of early growth response1 genes and their downstream target genes (Mishra et al., 2006). The observation that 1% CO only provided some protection at low doses of menadione challenge but not at higher doses (data not shown) plus the fact that no cytoprotective effect showed for biliverdin to bilirubin needs further investigation. One possibility is that delivering carbon monoxide in the environment rather than generating it intracellularly in the appropriate compartment as does heme oxygenase-1 may explain the fact that we did not obtain as complete a cytoprotective effect as compared to CAPE.

Many biological activities of polyphenols have been ascribed to their antioxidant properties. The 3,4 dihydroxyl configuration on the catechol ring of CAPE and similar polyphenolic compounds provides the key functional group for their free radical scavenging activity. Caffeic acid, a precursor and potential degradation product of CAPE, has been shown to possess similar antioxidant and free radical scavenging activities evaluated by three different methods including inhibitory effect on lipid oxidation by the rancimat method, scavenging effect on 2,2-diphenyl-L-picrylhydrazyl radicals, and antioxidative effect in oil-in-water emulsion (Chen and Ho, 1997). This was also confirmed with our cell-based antioxidant assay, showing CAPE and caffeic acid both reduced the basal intracellular level of reactive oxygen species to a significant level in 2 h. The result that caffeic acid showed no significant cytoprotection against menadione compared to CAPE indicates the transcription activity of these compounds may be more important than their direct antioxidant effect.

The HO-1 activation by plant-derived polyphenolic antioxidants has been observed in human smooth muscle cells by resveratrol (Juan et al., 2005), PC12 cells by carnosol (Martin et al., 2004), renal epithelial cells and endothelial cell by curcumin (Balogun et al., 2003; Motterlini et al., 2000). CAPE has also been identified as a HO-1 inducer in astrocytes (Scapagnini et al., 2002). The results reported here show that CAPE induces HO-1 in human endothelial cells. The major signaling transduction involved in HO-1 induction by those electrophilic polyphenolic compounds has been attributed to the activation of NF-E2 related factor-2 (Nrf2). Under normal conditions, Nrf2 is inactive in cytoplasm because it is bound to Keap 1. It has been suggested that these polyphenolic antioxidants interact with the thiol groups of Keap1, which releases Nrf2 from Nrf2/Keap1 complex. The free Nrf2 is subsequently translocated into the nucleus, where it forms heterodimers with small Maf, then binds to the antioxidant response element (ARE), and accelerates the transcription of HO-1 (Dinkova-Kostova et al., 2002). The activation of the Keap1/Nrf2/ARE pathway has been proposed to mediate the induction of other phase II detoxifying enzymes in addition to HO-1 including NAD(P)H: quinone oxidoreductase 1 and Glutathione *S*-transferases (Dinkova-Kostova et al., 2001). However, the gene response in HUVEC regulated by CAPE during the 6-h induction period didn't show any phase II genes induction other than HO-1 (Table 1). Although HO-1 up-regulation itself is sufficient to provide protection against oxidative stress (Otterbein et al., 2003), we cannot exclude the possibility that other detoxifying genes might be expressed earlier or later and exert protective effects.

However, a variety of other inducers of HO-1 chemically unrelated to polyphenols have been described (Kronke et al., 2003, 2007; Loboda et al., 2005; Prawan et al., 2008) and the mechanism of cytoprotection by way of biliverdin/bilirubin and carbon monoxide has come into question. Sheftel et al. (2007) have noted that nonheme inducers do not typically result in increases in intracellular ferritin as they do not provide a substrate for it and yet enzymatically inactive HO-1 transfected cells conferred cytoprotection (Hori et al., 2002). Lin et al. (2007) suggested that HO-1 may have the capacity to act as a transcription factor after its induction as it could be proteolytically

processed and translocated into the nucleus following synthesis. Some of the other genes upregulated by CAPE i.e. hsp70 and hsp90 that have been correlated with cytoprotection might be the actual protectants. Whether similar effects of HO-1 in human endothelial cells account for its cytoprotective effects is currently unknown.

In conclusion, the cytoprotective effect of CAPE in human endothelial cells, in large part, resulted from its transcriptional activities, mainly the induction of HO-1, rather than its structure-related antioxidant activity as caffeic acid exhibited good antioxidant activity but was not cytoprotective. The mechanism by which this relatively specific induction of HO-1 by CAPE in human endothelial cells is not entirely clear and may result from phosphorylation or other signaling events that are independent of the activation of the antioxidant response element that is commonly known to regulate the phase II response.

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